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Role of "*Dehalococcoides*" spp. in the Anaerobic Transformation of Hexachlorobenzene in European Rivers †

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The diffuse pollution by chlorinated organic compounds in river basins is a concern, due to their potential adverse effects on human health and the environment. Organohalides, like hexachlorobenzene (HCB), are recalcitrant to aerobic microbial degradation, and "*Dehalococcoides*" spp. are the only known microorganisms capable of anaerobic transformation of these compounds coupled to their growth. In this study, sediments from four European rivers were studied in order to determine their HCB dechlorination capacities and the role of *Dehalococcoides* spp. in this process. Only a weak correlation was observed between *Dehalococcoides* species abundance and HCB transformation rates from different locations. In one of these locations, in the Ebro River sediment, HCB dechlorination could be linked to *Dehalococcoides* species growth and activity by 16S rRNA-based molecular methods. Furthermore, HCB dechlorination activity in this sediment was found over the full range of ambient temperatures that this sediment can be exposed to during different seasons throughout the year. The sediment contained several reductive dehalogenase (*rdh*) genes, and analysis of their transcription revealed the dominance of *cbrA*, previously shown to encode a trichlorobenzene reductive dehalogenase. This study investigated the role of *Dehalococcoides* spp. in HCB dechlorination in river sediments and evaluated if the current knowledge of *rdh* genes could be used to assess HCB bioremediation potential.

Production of hexachlorobenzene (HCB)-containing pesticides is banned in most of the world due to HCB's toxic and carcinogenic nature. However, production and emission of the compound still occurs as an intermediate in chemical processes and from natural sources, such as volcanoes (5, 17). In aquatic environments, HCB is mainly deposited in the sediment due to its hydrophobicity. Bacterial reductive dechlorination plays an important role in the degradation of chlorinated aromatic contaminants like HCB in these anaerobic environments (15, 19). Under anaerobic conditions, HCB is used by some bacteria in their energy metabolism by coupling reductive dehalogenation to electron transport phosphorylation (36). This is the only known pathway, so far, for the microbial transformation of HCB that is linked to microbial growth. Reductive dechlorination of HCB and its lesser chlorinated derivatives was previously reported in several river sediments (8, 11, 18, 19). Nevertheless, in none of these studies were the microorganisms responsible for the process characterized.

Three strains of bacteria capable of degrading HCB via reductive dechlorination have been isolated so far. "*Dehalococcoides*" sp. strain CBDB1 and "*Dehalococcoides ethenogenes*" strain 195 dechlorinate HCB to 1,3-dichlorobenzene (DCB), 1,4-DCB, and 1,3,5-trichlorobenzene (TCB) (3, 14). Additionally, production of 1,2-DCB was also observed with strain 195. A distant relative of *Dehalococcoides* spp., "*Dehalobium chlorocoercia*" DF-1, dechlorinates HCB only to 1,3,5-TCB (48). Besides HCB, strain CBDB1 can also degrade polychlorinated dibenzodioxins, ethenes, phenols, and benzenes (1, 3, 10), whereas strain 195 can also use chlorinated ethenes and ethanes as electron acceptors (14, 31). Enzymes catalyzing the reductive dechlorination of HCB have not yet been identified. However, genomes of strains CBDB1 and 195 were shown to have multiple copies of putative reductive dehalogenase (*rdh*) genes that were predicted to code for enzymes mediating reductive dechlorination (29, 35). An *rdh* gene from strain CBDB1, *cbrA*, was recently characterized as a 1,2,3,4-tetrachlorobenzene (TeCB) and 1,2,3-TCB reductive dehalogenase gene (2).

The goal of the current study was to assess the HCB dechlorination potential of *Dehalococcoides* spp. in contaminated river sediments from different locations on the European continent and to evaluate if the current knowledge of *rdh* genes could be used to assess HCB bioremediation potential. Previous field surveys in several locations in two HCB-contaminated European rivers indicated that *Dehalococcoides* species activity and *rdh* gene content are variable between different geographical locations (42). Moreover, river water temperature was identified as one of the environmental parameters significantly affecting *Dehalococcoides* species composition in the river sediments. To this end, we used batch microcosm incubations to detect HCB dechlorination potentials in sediment samples from four different rivers. Moreover, the sediment from one location was exposed to the range of different temperatures to study the effect of this parameter on

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HCB dechlorination by *Dehalococcoides* spp. Changes in the *Dehalococcoides* species activity and diversity were detected via 16S rRNA-based approaches. Gene-specific PCR assays and sequencing were used to detect *rdh* gene diversity and expression.

MATERIALS AND METHODS

Origin of the river sediments. Samples were collected from four European rivers within the framework of the EU project Aquaterra (7). This project aimed to provide a foundation for a better understanding of the fate of pollutants in these river basins (for more information, see http://www.eu-aquaterra.de). The list of sampling locations is provided in Table S1 in the supplemental material. Some of the locations in these river systems have a known history of HCB contamination (e.g., Flix in the Ebro River basin), but almost all of them have been additionally contaminated with other chlorinated compounds, e.g., pesticides and/or chlorinated aliphatic compounds (7). River sediment and water samples were taken approximately 1.5 m away from the river shore. A closed 0.5- to 1-liter sterilized air-tight jar was submerged and opened just above the water-sediment interface. The jar was completely filled with top-layer sediment. The top 5 cm of the jar was filled with the river water, and the jar was closed before it was brought to the surface and transported to the laboratory in a cooler. Besides river sediment, samples from floodplain soil were also taken at the Elbe River. The sediments and water were stored at 4°C until further use. Sediment and soil characteristics have been reported elsewhere (42).

Chemicals. All chemicals were at least of analytical grade. HCB, 1,2,4,5-TeCB, 1,2,3,5-TeCB, 1,3,5-TCB, and 1,2-DCB were obtained from Aldrich Chemicals Co., Ltd. (Dorset, England). Pentachlorobenzene (QCB), 1,2,3,4-TeCB, 1,2,4-TCB, 1,3-DCB, and 1,4-DCB were purchased from Merck (Darmstadt, Germany). 1,2,3-TCB was from Janssen Chimica (Beerse, Belgium). All chemicals (purity, 99%) were used as received without further purification.

Batch experiments. The transformation of chlorinated benzenes was tested with 5 g (wet weight) of sediment in 120-ml glass bottles containing 50 ml basal medium specifically designed for the cultivation of reductively dechlorinating bacteria (0.1 g sediment/ml basal medium) as described previously, but without the addition of fermented yeast extract (20).The pH of the batches was 7.0 to 7.3. The gas phase consisted of N_2 (80%) and CO_2 (20%). The bottles were sealed with Viton stoppers (Maag Technic AG, Dubendorf, Switzerland). Lactate was added as an electron donor (final concentration, 50 mM), and 30 or 50 M HCB was added from a stock solution prepared in acetone. The resulting acetone concentration in the bottles was below 1 mM. Experiments were set up in an anaerobic glove box. The oxygen concentration in the glove box was kept low with a palladium catalyst and 3% H_2 in the gas phase, which otherwise consisted of N_2. The bottles were incubated statically at 30°C in the dark unless otherwise indicated. All experiments were carried out in triplicate. Sterile controls did not contain any sediment. Abiotic sediment controls were prepared by autoclaving the sediments twice for 20 min at 120°C at the start of the experiment. HCB dechlorination was not observed in the abiotic controls of the study (data not shown). Microcosms were sampled in intervals of 7 to 10 days to monitor dechlorination. Liquid phase extractions for chlorinated benzene measurements were performed on the sampling day.

Three different sets of batch scale experiments were conducted. (i) To test transformation of HCB in the sediments of four European rivers, samples taken from 2003 to 2006 were used. Within 2 to 4 weeks of sampling, batch experiments were established, except for sediments collected from the Rhine River. (ii) To assess the effect of laboratory medium compared to river water, samples that were collected in February 2006 from Flix in the Ebro River basin were used. The batch experiments were started within 2 weeks of sampling. In this experiment, two sets of bottles with river water as a growth medium were prepared with and without the addition of lactate as an external electron donor. River water controls without sediment addition were prepared to check if dechlorination occurs in the river water. (iii) Experiments that addressed the effect of temperature on HCB transformation were started in October 2006 with sediments collected in February 2006 from the Ebro River. The river sediments were subjected to temperatures in the range of 4 to 37°C.

Analytical methods. Total masses of chlorinated benzenes were determined by gas chromatography (GC)-mass spectrometry analysis of liquid phase samples. One microliter sediment culture slurry was extracted to 1 ml hexane-acetone (4:1 [vol/vol]) solution via sonication for 20 min, followed by overnight end-over-end shaking at room temperature. At least 95% of the added HCB could be recovered with this procedure. One microliter of the hexane-acetone solution was injected into a Trace DSQ System (Trace [Milan, Italy] DSQ MS Detector and Trace GC Ultra) equipped with an injection splitter (split ratio, 20:1) and an flame ionization detector (FID) connected to a capillary column (30 m by 0.25 m [inner diameter]; Rtx 5MS [0.25 mm thick]; Restek, PA). The carrier gas was helium, and the inlet pressure was 3 kPa. The operating temperatures of the injector and detector were 220 and 250°C, respectively. The column was operated with the following temperature program: initial column temperature, 60°C (1 min); increase of 5°C/min to a final temperature of 180°C (3 min). Output data were analyzed with the Trace (Milan, Italy) Xcalibur Data System 1.3. The chlorobenzenes (CBs) were quantified using 1,2-DCB and HCB as internal standards. For each bottle, duplicate measurements were done. As little as 2 M CB could be measured with this detection system. Graphs were drawn from averages of values measured in triplicate microcosms with corresponding standard deviations.

Sampling and nucleic acid extraction. DNA was isolated directly from sediment microcosms using the Fast DNASpinKit for Soil (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions. During the experiments, 1.5 ml sediment-liquid mixture per sampling point from Flix (Ebro River basin) sediment microcosms was stored at 80°C for nucleic acid extraction. RNA was extracted using the FastRNA Pro Soil-Direct Kit (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions with minor modifications (42).

16S rRNA-targeted DGGE, cloning, and sequencing and quantitative PCR. cDNA synthesis, PCR, denaturing gradient gel electrophoresis (DGGE), cloning, and sequence analysis were performed as described previously (41, 42). A complete list of primers used in this study is given in Table S2 in the supplemental material. Real-time quantitative PCR (RT-qPCR) was performed using an iQ5 iCycler (Bio-Rad, Veenendaal, The Netherlands) with the primers and thermocycling program as previously described (37) for 16S rRNA genes of *Dehalococcoides* and total *Bacteria* using SYBR green dye. For comparison, the 16S rRNA copy abundance of two other dechlorinating genera, namely, *Desulfitobacterium* and *Dehalobacter*, was also followed. Members of neither *Desulfitobacterium* nor *Dehalobacter* are currently known to be able to degrade HCB or its transformation products. All of the sampling points were analyzed in triplicate, and no-template controls were included. Regressions with r^2 values of at least 0.994 were fitted to the data. The amplification efficiency was 99 to 103% for 16S rRNA- and 16S rRNA gene-targeted assays and 103 to 108% for reductive dehalogenase-encoding gene-targeted assays. As a measure of the relative abundance of active dechlorinating bacteria, the percentage of 16S rRNA copies of dechlorinating bacteria compared to the number of total bacterial 16S rRNA copies (e.g., [*Dehalococcoides* species 16S rRNA copies/total *Bacteria* 16S rRNA copies] 100) was calculated. The growth yield of *Dehalococcoides* spp. was calculated as the ratio of 16S rRNA gene copies/ml to the amount of Cl per ml released during HCB transformation at the point of HCB depletion by assuming (i) *Dehalococcoides* spp. are the only microorganisms responsible for HCB transformation and (ii) *Dehalococcoides* spp. carry only one copy of the 16S rRNA gene in their genomes.

Detection of reductive dehalogenase genes. Terminal restriction fragment length polymorphism (T-RFLP), sequencing, and qPCR were used to detect *rdh* genes in the genome of strain CBDB1 in sediment microcosms. As described previously by Wagner and coworkers, 13 degenerate primer pairs were used to amplify specific fragments of catalytic-subunit-encoding *rdh* genes (*rdhA*) from strain CBDB1 (44). T-RFLP analysis of 6-carboxyfluorescein (FAM)-labeled fragments amplified from the sample taken in the third week of HCB dechlorination in Flix sediment microcosms was performed by Dr. Van Haeringen Laboratorium b.v. (Wageningen, The Netherlands). Additionally, degenerate primers (forward primer, fdehal, and reverse primer, rdehal [see Table S2 in the supplemental material]) were used to amplify 550- to 660-bp fragments. These primers target conserved motifs in the upstream region of the iron-sulfur cluster binding motif of *rdhA* genes in strain CBDB1 (21). After cloning and sequencing of these PCR fragments, the sequences obtained were compared to those deposited in the National Center for Biotechnology Information (NCBI) database using the BLASTX tool (4). Deduced amino acid sequences were obtained with the TRANSLATE program (http://us.expasy.org/tools/dna.html). Amino acid sequences were aligned using the MUSCLE program (http://www.ebi.ac.uk/Tools/muscle/), and phylogenetic trees (neighbor joining; default settings) were generated by using MEGA version 4.0 (40). cDNA for the detection of *rdh* gene expression was synthesized with random hexamers from 0.1 g of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The expression of two reductive dehalogenase-encoding genes of strain CBDB1, namely, *cbrA* (CAI82345.1) and cbdbA1624 (CAI83644.1), was followed in experiments addressing the effect of temperature on HCB transformation via RT-qPCR using an iQ5 iCycler (Bio-Rad, Veenendaal, The Netherlands) with the primers and thermocycling program as previously described (44). *cbrA* is a 1,2,3,4-tetrachlorobenzene and 1,2,3-trichlorobenzene dehalogenase gene. Furthermore, Wagner and coworkers

TABLE 1. Summary of results from batch biotransformation experiments and quantitative PCR analysis

Location	Nature[a]	Biotransformation expt, HCB			Quantitative PCR analysis[e]	
		$t_{1/2}$ (days) (n^b)	Lag phase[c] (days)	Product[d]	*Dehaloccoides* spp. (10^2 16S rRNA gene copies/ml)	*Bacteria* (10^6 16S rRNA gene copies/ml)
Ebro River						
Reinosa	RS	(3)	ND[1/2]	ND	BDL	0.04 0.01
Conchas de Haro	RS	7–30 (3)	5–48	1,3,5-TCB, 1,3/1,4-DCB	BDL	4.58 1.81
Zaragoza	RS	3–27 (3)	5–19	1,3,5-TCB, 1,3/1,4-DCB	1.46 0.5	1.41 0.03
Monzon	RS	5–6 (2)	28	1,3,5-TCB, 1,3/1,4-DCB	2.00 0.1	0.63 0.05
Lerida	RS	14–30 (2)	28	1,3,5-TCB, 1,3/1,4-DCB	6.23 0.3	3.85 0.67
Flix	RS	2–47 (4)	28	1,3,5-TCB, 1,3/1,4-DCB	3.15 0.16	0.42 0.05
Tortosa	RS	6–12 (2)	28	1,3,5-TCB, 1,3/1,4-DCB	5.38 0.21	1.68 0.51
Delta rice Þeld	RS	5–62 (4)	36	1,3,5-TCB, 1,3/1,4-DCB	2.30–4.61 0.1–0.2	2.27 0.28
Delta-estuary	RS	8–11 (2)	28	1,3/1,4-DCB	0.86 0.14	1.87 0.86
Elbe River						
Schönberg	RS	6–17 (3)	62	1,3,5-TCB, 1,3/1,4-DCB	BDL–2.15 0.1	1.96 0.12
Schönberg-42	FPS	(2)	ND	ND	BDL	5.33 0.37
Schönberg-45	FPS	6–140 (3)	28–62	1,2,4/1,3,5-TCB, 1,2-DCB	BDL	5.45 0.88
Roßlau	RS	(4)	ND	ND	1.23 0.1	0.35 0.15
Other sampling locations						
Danube River, Budapest	RS	4–64 (3)	5–19	1,3,5-TCB, 1,3/1,4-DCB	1.92 0.11	1.35 0.15
Rhine River, Wageningen	RS	6–14 (2)	28	1,3,5-TCB, 1,2/1,4-DCB	2.07 0.1	1.01 0.06

[a] RS, river sediment; FPS, ßood plain soil.
[b] Calculated half-life (via Þrst-order rate constants) *n*, number of bottles used for the calculation.
[c] Observed lag phase before dechlorination occurred.
[d] Major chlorinated benzene products after 5 months.
[e] Mean value and standard deviation of (at least) triplicate analysis. BDL, below detection limit.
[f] ND, no transformation could be detected in the experimental period of 153 days.

(44) showed that cbdbA1624 is expressed at levels similar to those of *cbrA* by strain CBDB1 in the presence of 1,2,4-TCB. Expression of both *cbrA* and cbdbA1624 was found to be induced—compared to the other *rdh* genes of strain CBDB1—during 1,2,3-TCB and/or 1,2,4-TCB dechlorination.

Nucleotide sequence accession numbers. The sequences obtained were deposited in the NCBI database under accession numbers HQ008326 to HQ008336.

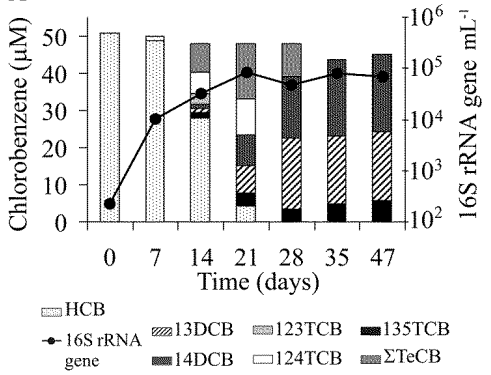
RESULTS

Dechlorination of HCB in river sediments from different geographical locations. The primary objective of this survey was to test the anaerobic transformation potential of HCB in sediments of different rivers across Europe. Twelve out of 15 sediments were able to transform HCB to lower chlorinated benzenes (Table 1). HCB was not degraded in abiotic or autoclaved controls, indicating that microbial transformation was responsible for the removal of chlorinated benzenes. Penta- or tetrachlorobenzenes were always below the detection limit, suggesting that these intermediates were dechlorinated rapidly. Monochlorobenzene (MCB) formation was not observed during the 150 days of incubation. The electron donor was completely consumed, and methane accumulation was observed (data not shown). In most cases 1,3,5-TCB and 1,3- and 1,4-DCB were the end products observed after (up to 5 months) incubation. In Schönberg-45 soil and river sediment from Wageningen, 1,2-DCB was also an end product of the transformation (Table 1). Samples from Reinosa, Schönberg-42 soil, and Roßlau were inactive toward HCB, but methanogenic activity was detected in these samples. Lack of dechlorination activity in the Roßlau samples could be due to the large amount of heavy metal deposition in this river (28).

Dehalococcoides was detected in most, but not all, of the samples studied here (Table 1). In most of the samples exhibiting HCB-dechlorinating activity in microcosms, *Dehalococcoides* was present, but in some cases, i.e., Conchas de Haro and the Schönberg ßood plain soils, *Dehalococcoides* could not be detected in the sediment or soil prior to the experiments, although HCB dechlorination was observed. The *Dehalococcoides* species 16S rRNA gene copy number per gram of Reinosa, Roßlau, and Schönberg-42 material was below the detection limit, which may explain the lack of transformation by these particular samples. The relative abundance of *Dehalococcoides* species 16S rRNA gene copies was mostly below 0.1% compared to the total *Bacteria* 16S rRNA gene copies. The data from the HCB transformation experiments was used to determine HCB dechlorination rates, where Þrst-order rate constants were calculated from the HCB transformation curves (Table 1). A large variation was observed in degradation rates among the samples from each basin (see Fig. S1 in the supplemental material). These transformation rates had a weak negative correlation (Spearman's rho 0.33; P 0.1) with the number of *Dehalococcoides* organisms in the samples, suggesting that the initial *Dehalococcoides* species content of the samples is not necessarily indicative of HCB transformation rates. Short half-lives of only a few days were observed for sediments at some locations. One of these, Flix in the River Ebro basin, Spain, was selected for follow-up experiments.

Dechlorination of HCB in river sediment from Flix in the Ebro River basin. Flix sediments were amended with 50 M HCB to gain additional insight into HCB transformation and the role of *Dehalococcoides* spp. in the process. HCB was completely transformed in all microcosms to 1,3,5-TCB, 1,4-

A



B

Gene Clusters	Number of genes in each cluster	Detected	Not Detected
cluster 1a	5	cbdbA1453, 1550, 1570, 1595, 1624	
cluster 1b	2	cbdbA1495, 1508	
cluster 1c	4	cbdbA1588, 96, 1582, 1535	
cluster 1d	3	cbdbA1546, 1575	cbdbA1578
cluster 2a_1	2	*cbrA* (cbdbA84), 1563	
cluster 2a_2	1	cbdbA1638	
cluster 2a_3	2	cbdbA1560	cbdbA1627
cluster 2b_1	2		cbdbA1542, 1491
cluster 2b_2	2		cbdbA238, 1455
cluster 2b_3	2		cbdbA1598, 1618
cluster 3	3		cbdbA80, 88, 243
cluster 4a	2	cbdbA1503, 1092	
cluster 4b	2		cbdbA187, 1539

FIG. 1. (A) Transformation of 50 M HCB in river sediment microcosms from Flix (Ebro River basin, Spain). The bars represent the concentrations of HCB and transformation products. The line represents *Dehalococcoides* species 16S rRNA gene copies/ml (standard deviations are not displayed if they are smaller than the symbol). (B) T-RFLP Þngerprinting of *Dehalococcoides* sp. strain CBDB1 reductive dehalogenase (*rdh*) genes in sediment microcosms. The gene clusters are as previously deÞned in Table 1 of reference 44 and represent the degenerate primer pairs used to amplify speciÞc fragments of all 32 *rdhA* genes of strain CBDB1.

DCB, and 1,3-DCB (Fig. 1A). Throughout the experiment (91 days), MCB production was not detected. Additionally, no changes could be detected in tri- or dichlorobenzene concentrations after the 28th day of the experiment (data not shown). Interestingly, 16S rRNA gene copy numbers of *Dehalococcoides* spp. increased rapidly to 10^4 copies/ml in the Þrst week of the experiment where no HCB dechlorination was observed. Dechlorination proceeded with an additional further increase in 16S rRNA gene copy numbers to 10^5 copies/ml, which was reached after 3 weeks (Fig. 1). Similar population densities were also observed in *Dehalococcoides*-containing enrichment cultures and isolated strains (13, 22, 23, 31). Besides absolute 16S rRNA gene copy numbers, rRNA relative abundance (*Dehalococcoides* species 16S rRNA copies/total *Bacteria* 16S rRNA copies) was used to relate the growth of the species to the transformation of HCB. Even though the rRNA content of metabolically active or inactive *Dehalococcoides* spp. is not known, a strong positive correlation between activity and high rRNA content has been shown for some other microorganisms (24, 25). Over the duration of HCB transformation, the relative abundance of *Dehalococcoides* species rRNA compared to total bacterial rRNA levels increased from 0.7 to 5.7%. This value rapidly decreased again after HCB transformation was complete (see Fig. S2 in the supplemental material). There was no substantial change in the relative abundance of *DesulÞtobacterium* species rRNA (less than 0.1% during the experimental period [data not shown]), and *Dehalobacter* species rRNA was not detected at any time (data not shown). The measured *Dehalococcoides* species growth yield was 3.61 10^6 0.5 10^6 16S rRNA gene copies/ mol Cl released. *Dehalococcoides* sp. strain CBDB1 and strain 195 were reported to have growth yields 17 and 23 times higher, respectively, than this value during dechlorination of dichlorophenol to monochlorophenol (1).

Dehalococcoides species 16S rRNA-targeted DGGE analysis showed no changes in the composition of the *Dehalococcoides* species population during dechlorination (see Fig. S3A in the supplemental material). The dominant DGGE band corresponded to a single *Dehalococcoides* strain (EU700498), the 16S rRNA sequence of which had 98.1% (1,200 bp) similarity to that of *Dehalococcoides* sp. strain CBDB1. In addition, during the dechlorination of HCB and its intermediate products, no signiÞcant changes were detected in the total bacterial DGGE Þngerprints targeting the 16S rRNA gene (see Fig. S3B in the supplemental material).

We used T-RFLP to assess whether the *Dehalococcoides* spp. in Flix river sediment possessed *rdhA* genes corresponding to those previously identiÞed in the genome of strain CBDB1 (44). Analysis of 57 T-RFs in the sample taken in the third week of the experiment showed that the Flix sediment batches contained 19 of the 32 *rdh* genes from strain CBDB1 (Fig. 1B). Almost all of the *rdh* gene sequences belonging to clusters 1a to d, 2a, and 4a were detected. Sequencing of gene fragments (550 to 660 bp) ampliÞed with the fdehal-rdehal primer pair resulted in 11 unique sequences. Deduced amino acid sequences of partial *rdhA* gene sequences from Flix microcosms (184 to 221 residues) were used to generate a tree in which *rdhA* genes from *Dehalococcoides* species isolates with at least 70% similarity to Flix microcosm sequences were also included (Fig. 2). The recently characterized trichlorobenzene reductase-encoding gene, *cbrA*, could be detected with T-RFLP analysis but could not be found in the clone library. In contrast, two genes that could not be detected by T-RFLP analysis (cbdbA1455 and cbdbA1618 of cluster 2b) were found in the clone library. Sequences clustered in several groups. Cluster 1 contained four sequences similar to cbdbA1624 alongside several uncharacterized *rdhA* genes from *Dehalococcoides* species strains KB1, GT, and VS and putative *trans*-dichloroethene-producing reductive dehalogenase from *Dehalococcoides* sp. strain MB (12). Cluster 2 comprised *rdhA5* from *Dehalococcoides* sp. strain FL2 and an uncharacterized *rdhA* gene from strain GT, besides four sequences from Flix microcosms with highest similarity to cbdb1560. The deduced amino acid sequences of RdhA01, RdhA04, and RdhA09 contained at least one stop codon, suggesting that these genes are likely to be nonfunctional.

Dechlorination of HCB in Flix sediment with river water as a growth medium. The Flix sediment was subjected to river water instead of the deÞned laboratory medium to test whether HCB transformation could be achieved. In all of the river sediment batches, methane production could be observed 20

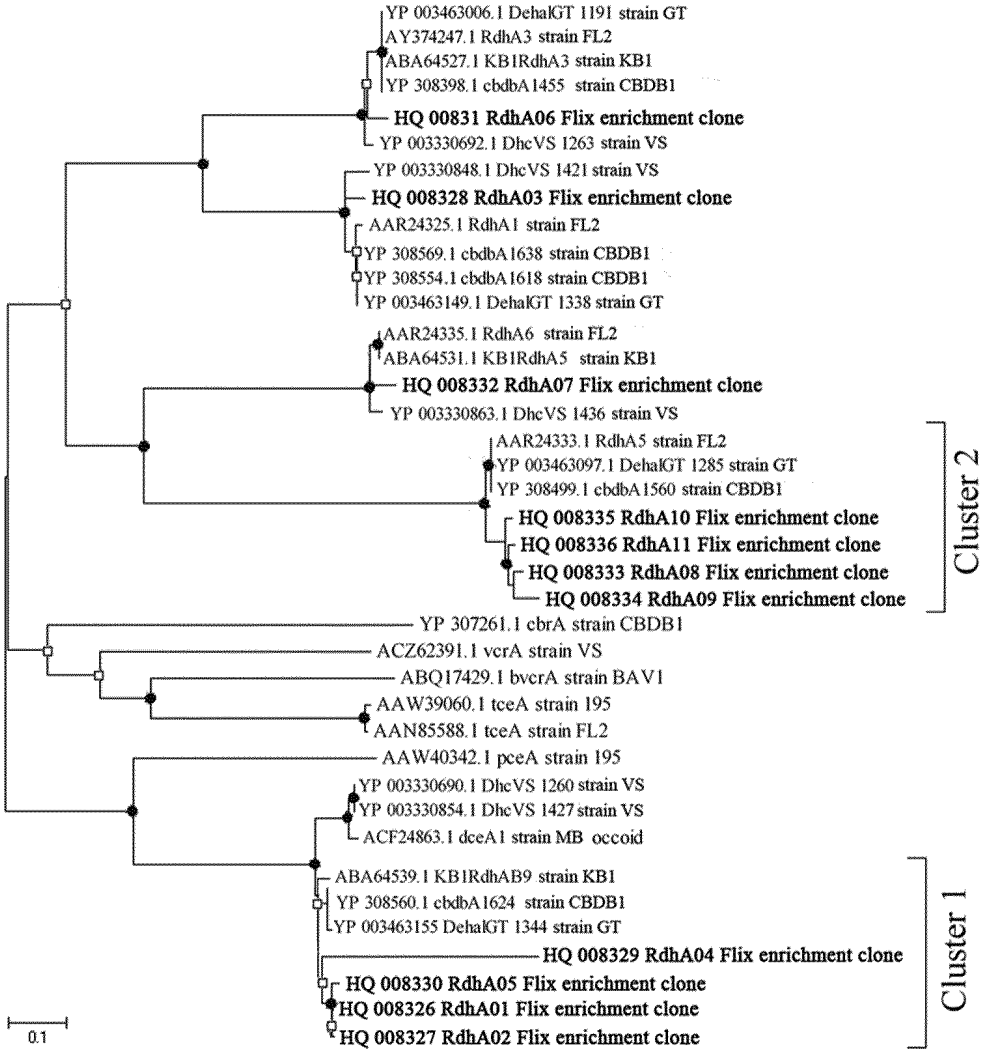


FIG. 2. Phylogenetic analysis of *rdhA* genes amplified from HCB-dechlorinating sediment microcosms from Flix in the Ebro River basin. The unrooted neighbor-joining tree was generated from deduced amino acid sequences of partial *rdhA* gene fragments. Branching points supported by 85 to 100% of 1,000 bootstrap sampling events are indicated by solid circles, and those with 50 to 84% support are indicated by open squares. The scale bar represents 10% sequence divergence. Distances were computed using the Poisson correction method. All positions containing gaps and missing data were eliminated from the data set. The labels show the GI number of the gene, the locus tag of the coding sequence, and the taxonomic name of the *Dehalococcoides* strain. Genes sequenced in this study are presented in boldface.

days after the start of the experiment (data not shown), indicating the development of methanogenic conditions. HCB dechlorination in bottles with lactate started after 10 days, whereas bottles without an external electron donor had a lag phase of 30 days before dechlorination could be observed (see Fig. S4A and B in the supplemental material). Full dechlorination of HCB was achieved in both cases. The end products were similar to those observed in the previous experiment with defined medium, namely, 1,3,5-TCB, 1,3-DCB, and 1,4-DCB. However, more 1,3,5-TCB was produced (compare Fig. 1A with Fig. S4A and B in the supplemental material). No dechlorination was observed in river water controls without sediment. Comparison of DGGE fingerprints showed that active species in the sediment batches had the same fingerprint as *Dehalococcoides* sp. CBDB1 (see Fig. S4C in the supplemental material). On the other hand, these fingerprints were different than those from the predominant endogenous populations observed in the original sediment samples from the sampling location. Molecular screening showed that the activity of *Dehalococcoides* spp. (based on 16S rRNA) increased during dechlorination, covering 0.5 to 0.9% of the bacterial 16S rRNA pool, in the absence and presence, respectively, of the external electron donor (see Fig. S4D in the supplemental material). Bottles with the external electron donor showed a pattern of activity similar to that observed in the bottles with a defined medium. On the other hand, incubations without an external electron donor showed a more gradual increase and decrease in the 16S rRNA copy numbers, as well as a 2-fold-lower maximal relative abundance.

Dechlorination of HCB at different temperatures. In order to assess whether seasonal differences in ambient temperature have an effect on HCB transformation, the Flix river sediment was subjected to temperatures between 4 and 37°C. Transformation of 30 M HCB was observed at all tested temperatures,

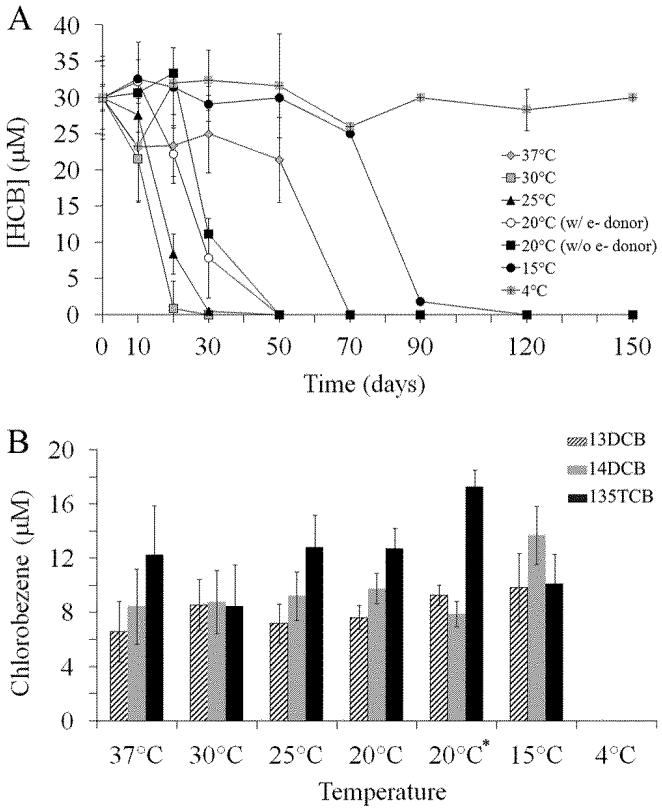


FIG. 3. (A) Transformation of HCB at various temperatures (4 to 37°C) in Flix river sediment. "20°C w/e- donor" represents the batch microcosm at 20°C with lactate as an electron (e) donor. "20°C w/o e- donor" represents the batch microcosm at 20°C without any additional e donor. (B) HCB transformation products at the end of the experiment (150 days). The asterisk represents the batch microcosm at 20°C without any additional e donor. The data points are averages of duplicate measurements of triplicate bottles. The error bars represent the standard deviations between the triplicate microcosms.

except for 4°C (Fig. 3A). The highest HCB transformation rates were observed at 25°C and 30°C, whereas at temperatures below 20°C or above 30°C, the dechlorination rate dropped considerably (Fig. 3A). The results indicated that it was still possible to have reductive dechlorination at low temperatures, yet the time needed for complete degradation almost doubled (from 50 days at 20°C to more than 100 days at 15°C). Similar to the results obtained in the river water microcosms, at 20°C, the lack of an added external electron donor slightly delayed the start of HCB dechlorination. Interestingly, more 1,3,5-TCB was produced in microcosms without an added electron donor (Fig. 3B). There were no prominent differences between the end products of the transformation process at 30°C and 15°C (Fig. 4). 16S rRNA gene copies of *Dehalococcoides* spp. increased with HCB depletion at 30°C. On the other hand, at 15°C *Dehalococcoides* species copy numbers increased gradually without a signiÞcant change in HCB concentrations. Additionally, the relative abundance of *Dehalococcoides* species 16S rRNA increased to its highest level (5.2% 1.0% at 30°C and 5.5% 1.2% at 15°C) (see Fig. S5 in the supplemental material) during HCB dechlorination. DGGE Þngerprinting showed that at both temperatures the *Dehalococcoides* species 16S rRNA composition was the same in the batches, dominated by a single band (data not shown).

To further investigate the involvement of speciÞc reductive dehalogenases in the transformation of HCB, we also measured transcript levels of 1,2,3,4-TeCB and 1,2,3-TCB reductase-encoding *cbrA* and cbdbA1624, the latter of which is potentially involved in 1,2,4-TCB degradation (44). Transcript copy numbers were normalized to 16S rRNA and examined during the transformation of HCB at the two different temperatures (Fig. 5). At both temperatures, transcription of *cbrA* could be detected from the early weeks of the experiment, whereas transcription of cbdbA1624 could be observed only during the later phases of dechlorination. At 30°C, the relative transcript levels of *cbrA* increased by an order of magnitude compared to the beginning of the experiment (Fig. 5A). A similar response was observed during 1,2,3-TCB and 1,2,4-TCB dechlorination by strain CBDB1 (44). Throughout the experiment, *cbrA* transcript levels were found to be 3- to 50-fold higher than those at the start of the incubation. When transcribed, the expression of cbdbA1624 was lower than that of *cbrA*. At 15°C, transcription of the two genes was not detected at the beginning of the experiment. Later, *cbrA* was transcribed at a constant level until the transformation of HCB was set off. The *cbrA* transcription increased by only 1 order of magnitude once HCB transformation and accumulation of intermediates could be observed (Fig. 5B). On the other hand, cbdbA1624 did not follow a particular pattern, and its transcription level remained lower than that of *cbrA* (except for the sample taken at day 20 of incubation). Interestingly the

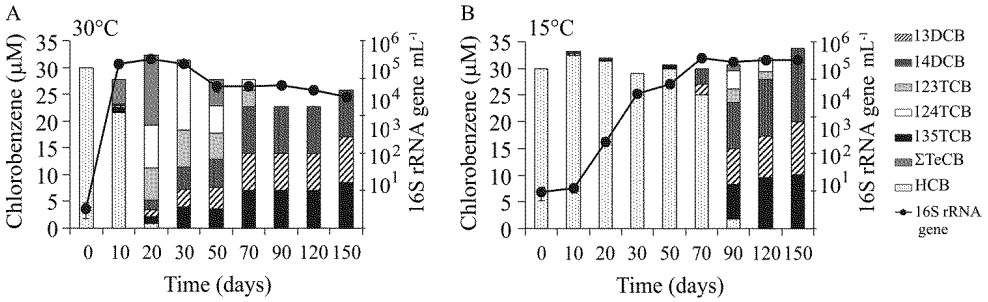


FIG. 4. Transformation of 30 M HCB in microcosms incubated at 30°C (A) and 15°C (B). The bars represent changes in the concentrations of HCB and the end products of transformation (with an average standard deviation of 5 M). The lines represent *Dehalococcoides* species 16S rRNA gene copies/ml. The error bars represent the standard deviations of duplicate measurements (standard deviations are not displayed if they are smaller than the symbol). A lack of data points indicates that concentrations were below the detection limit.

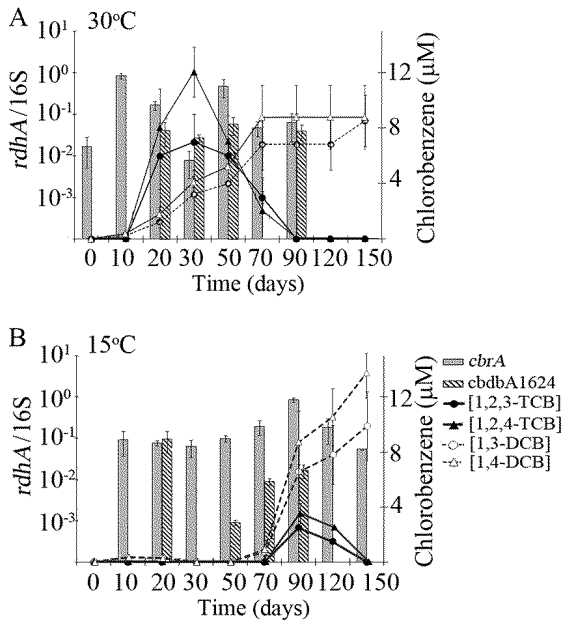


FIG. 5. Expression of *cbrA* and cbdbA1624 genes at 30°C (A) and 15°C (B) in sediment microcosms. Normalization was based on 16S rRNA copies. The bars represent the ratio of each *rdh* transcript to 16S rRNA. The error bars represent the standard deviations of duplicate measurements. When not detected, the gene expression in the samples is not displayed. The lines represent changes in HCB concentrations; The error bars represent average measurements of triplicates.

cbdbA1624 transcription level increased in the later stages of the experiment where 1,2,4-TCB dechlorination was observed. Subsequently, the number of *Dehalococcoides* 16S rRNA copies present in batch cultures was compared to the sum of *cbrA* and cbdbA1624 transcript copies. This comparison was made to determine whether a positive linear correlation existed between 16S rRNA and *rdh* gene transcription, as an indication of overall metabolic activity in relation to *rdh* transcription (see Fig. S6 in the supplemental material). A strong positive linear correlation (r^2 0.969) was found in the microcosms at 15°C, whereas a weaker correlation (r^2 0.636) was observed at 30°C.

DISCUSSION

In this study, we used a combination of molecular tools with chemical analysis to determine the role of *Dehalococcoides* spp. in the transformation process of HCB in river sediment microcosms. The majority of samples tested exhibited the capacity to dechlorinate HCB irrespective of the amount of HCB or *Dehalococcoides* spp. present *in situ*. The relatively widespread capability of sediments and soils to transform HCB is remarkable but not entirely unexpected. Although the amount of HCB in the environment has been decreasing over the past 3 to 4 decades, there still remains a substantial amount (between 10,000 and 26,000 tons worldwide) due to the global turnover of HCB (6). Our previous studies on these sediments indicated that the *in situ Dehalococcoides* composition is highly heterogeneous and cannot always be signiÞcantly correlated with HCB contamination (42). A similar conclusion could be drawn from the present study, as we observed only a weak correlation between *Dehalococcoides* species abundance in sediments and the rate at which the transformation of HCB took place in microcosms. Therefore, it could be postulated that not all of the *Dehalococcoides* spp. detected in sediments were capable of transforming HCB and/or that other populations not detected by the assays used in this study are at least partially responsible for HCB dechlorination. The 16S rRNA gene-based detection of *Dehalococcoides* used here overlooks the involvement of populations like strain DF-1, Lahn, and Tidal Flat clusters (26, 27), which are closely related to but not within the genus *Dehalococcoides*. It should be noted, however, that the current data set is small from a statistical viewpoint and may be too small to observe signiÞcant and/or strong correlations.

Even though dechlorination capacity and *Dehalococcoides* spp. were present in the samples, HCB contamination is still prevalent in the environment. This is not entirely unexpected, since the transformation experiments described here were carried out under optimized conditions for the dechlorinating bacteria, e.g., the medium contained, to the best of our knowledge, every nutrient the microorganisms might require. For example, as nonfermentative bacteria, *Dehalococcoides* spp. depend in the environment on the H_2 supplied by other microorganisms (38). Conversely, our results also showed that river water and sediment microorganisms could support the growth and dechlorination activity of *Dehalococcoides* spp. This discrepancy with the real-life situation of HCB still being present in the sediment indicates that prevailing environmental conditions often do not allow complete natural attenuation of HCB by *Dehalococcoides* spp. in contaminated sediments. However, compared to chemical (e.g., chemical reaction with hydroxyl [OH] radicals, with a half-life of 156 days to 4.2 years) or physical (e.g., atmospheric photolysis, with a suggested half-life of about 80 days) processes, biological reductive dechlorination of HCB with the half-lives observed in this study, ranging from 2 to 62 days, is a relatively fast process, provided that the conditions are suitable for dechlorination. Moreover, the results presented in this study support the Þndings of recent multimedia fate models, which predict that, on a global scale, the greatest losses of HCB in the environment may occur from sediment and soils (6).

SigniÞcant changes in the 16S rRNA relative abundance and 16S rRNA gene copy numbers of *Dehalococcoides* spp. were observed in all microcosms during HCB dechlorination. The results suggested the involvement of *Dehalococcoides* spp. in reductive dechlorination of HCB, while 16S rRNA sequence analysis conÞrmed the high sequence identity of *Dehalococcoides* spp. from the microcosm to strain CBDB1. We observed an increase in *Dehalococcoides* 16S rRNA gene copies before HCB transformation, which contradicts the recent knowledge that *Dehalococcoides* spp. can only use chlorinated compounds as their electron acceptor. Sequenced *Dehalococcoides* genomes have shown no strong indication of the presence of genes encoding enzymes involved in the use of any other electron acceptors (29, 32, 35). However, the sequenced species were enriched and maintained in the laboratory for a long time solely on chlorinated compounds and might have lost their ability to use other compounds. Even though we lack sound experimental proof, it is tempting to speculate, based on this observation, that *Dehalococcoides* might be able to use other

naturally produced (chloro)organic compounds, such as humic acids, as the electron acceptor in these river sediments. This should be addressed in future studies, aiming at a more profound understanding of the *in situ* ecophysiology of *Dehalococcoides* spp.

Experiments showed that, like the laboratory isolates, naturally occurring *Dehalococcoides* spp. are mesophilic and can tolerate 15 to 20°C environments, as well. Decreasing temperature caused a significant delay in the dechlorination of HCB and growth of *Dehalococcoides* spp. Our results also showed that *Dehalococcoides* spp. in the Flix sediment are not adapted to temperatures as low as 4°C. Tetrachloroethene (PCE) and polychlorinated biphenyls (PCBs) can be transformed at low temperatures (4 to 12°C) (9, 33); however, the involvement of *Dehalococcoides* spp. in these transformations is not known. There was no notable difference in the composition and relative abundance of the genus between 30°C and 15°C. It can therefore be assumed that below the optimum growth temperature of *Dehalococcoides* spp., HCB dechlorination is possible, with longer lag phases. Moreover, this experiment could not confirm our prediction from *in situ* surveys that temperature has a significant effect on *Dehalococcoides* species composition. The *Dehalococcoides* species population involved in HCB dechlorination in the microcosm showed 95.5 to 98.2% 16S rRNA sequence similarity to *Dehalococcoides* spp. detected in the sampling location (42) and 98.1% similarity to strain CBDB1. However, it should be kept in mind that the conditions used for *in vitro* microcosm experiments are selective and might not be ideal to support the growth of all endogenous *Dehalococcoides* species populations, which are involved in HCB transformation in these sediments at different seasons throughout the year.

The *Dehalococcoides* species 16S rRNA gene is highly conserved among currently known species. However, there are considerable differences in *rdh* gene contents (30). HCB transformation end products in microcosms were most similar to those reported for strain CBDB1. For this reason, we have focused on *rdh* genes from this strain. PCR assays targeting all *rdh* genes predicted from the genome sequence of strain CBDB1 showed the presence of 19 *rdh* genes in the microcosm. However, efforts to confirm this finding with sequencing were not successful. Our repeated attempts to amplify full-length fragments of *rdhA* genes (1.7 kb) with the primer pair RRF2-B1R (21) did not result in any PCR product. Even though the majority of known *rdh* genes from *Dehalococcoides* spp. were amplified using this primer set (21, 39), it also failed to amplify some *rdh* genes (21, 45). Additionally, in the full genome sequence of strain CBDB1, there are only four *rdhA* genes that have a perfect binding site to these primers. Sequencing of partial *rdhA* genes with the fdehal-rdehal primer pair showed conflicting results by amplifying two genes that could not be detected with T-RFLP analysis and showing low *rdh* gene diversity. The fdehal-rdehal primer pair has binding sites in two *rdhA* genes of strain CBDB1, cbdbA1495 and cbdbA1550; however, sequences obtained from the Flix microcosms showed only moderate similarity to these genes. The high overall sequence variability of *rdhA* genes required the use of degenerate primer pairs, but primer degeneracy introduces difficulties in detecting highly variable or low-abundance sequences (44). Nevertheless, we were able to gain a significant amount of information about *rdh* gene content and transcription in the microcosms. Flix sediment microcosms contained multiple copies of *rdh* genes, as previously shown for *Dehalococcoides* isolates and enrichments. Moreover, most of the genes detected via T-RFLP and sequencing analysis in the microcosms were also found *in situ* with functional gene microarrays (42). The 16S rRNA concentrations and *cbrA* transcription increased concurrently during dechlorination, supporting previous reports on the metabolic involvement of this gene in one of the HCB degradation pathways (44). The transcription of *cbrA* in the early phases of the degradation could also be an indication of the involvement of the gene in dechlorination of highly chlorinated benzenes. However, *cbrA* was consistently transcribed in most of the microcosms even after dechlorination was complete. As a result, substrate specificity and/or tight transcriptional regulation of the gene in our case is uncertain. Transcription of cbdbA1624 could not be correlated with *Dehalococcoides* growth and was observed only when HCB dechlorination had already started. Lack of expression during early HCB dechlorination supports the idea that the corresponding gene product is involved in dechlorination of lower chlorinated benzenes. Interestingly, at 15°C, *rdh* gene transcripts showed a stronger correlation with 16S rRNA copy numbers than at 30°C. Therefore, it can be speculated that at mesophilic temperatures, besides *cbrA* and cbdbA1624, some other *rdh* genes contribute significantly to the metabolic activity. Complex transcriptional responses to various chlorinated compounds were previously reported for *Dehalococcoides* isolates and cocultures (16, 44, 45) and were predicted to be tightly regulated by two-component and/or MarR-type regulators (29, 35). The majority of *rdh* genes detected with T-RFLP and sequencing analysis were similar to those of strain CBDB1, which are located close to MarR-type regulators. These regulators were shown to have "phenolic-sensing capabilities" (47) and to activate or repress gene expression during aromatic-compound degradation (43) in several other bacteria. It has been suggested that the possibly MarR-regulated *rdhA* genes might play an important role in haloaromatic compound degradation pathways (29). Since these genes could be detected both *in situ* and in sediment microcosms, it is tempting to speculate that they carry a great potential to be used as biomarkers of anaerobic haloaromatic-compound degradation capacity.

Conclusions. The HCB transformation capability of the river systems studied here is a promising indicator of their *in situ* capability for pollutant biodegradation. More environmental surveys are still needed to fully understand the degradation of halogenated aromatic pollutants, like HCB, in river basins. As strictly anaerobic and mesophilic bacteria, *Dehalococcoides* spp. were shown to take part in HCB transformation and to be resistant and adaptable to different temperatures. Our results suggest that similarities in *rdh* gene content and 16S rRNA-based identity can be used to assess HCB dechlorination capability and as possible indicators of degradation pathways. However, it is also evident that with biomolecular assays targeting ribosomal and process-specific functional genes, such as those encoding reductive dehalogenases, it will remain difficult to understand the full extent of the process, since the dechlorination process is part of a complex web of metabolic and regulatory interactions (34, 46). Future efforts focusing on

gene trait matching and elucidation of structure-function relationships for possibly MarR-regulated *rdhA* genes of *Dehalococcoides* spp. will further improve the predictive power of these molecular analyses.

ACKNOWLEDGMENTS

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